Synthetic analogues.

The following synthetic peptide analogues to IT9302 were synthetized by Professor Arne Holm at The Royal Veterinary and Agricultural University, Copenhagen, and tested by us for their ability to induce IRAP in cell cultures, which was measured by ELISA (Quantikine Immunoassay, Human IL-1ra DR00, R&D Systems, UK).

Experimentel conditions.

Peptides were reconstituted in sterile filtrated resolution buffer PBS pH 7.4 and /or PBS pH 7.4 with 4 % BSA (Sigma, A-9647). Thereafter 1 x 10^6 - 2 x 10^6 purified monocytes were stimulated with 0, 1, 10 and 100 ng / ml peptides diluted to ekvimolar concentration to rhIL-10, in RPMI with 2 % Fetal calf serum for 24 hours. (FCS, Noth American, Life technology, cat Nr. 16000044)

The list of synthetic peptides which were tested:		MW Da
Original peptide:	H - A Y M T M K I R N -OH	1127
Analogues:		
	H-**MEA** - Y M T M K I R N - OH	1171.3
	H-**E - MEA** - Y M T M K I R N -OH	1300.4
	H-**1BUA** - Y M T M KI R N - OH	1169.3
	H-**E - 1BUA** - Y M T M K I R N - OH	1298.5
	H-**E** A Y M T M K I R N - OH	1256.5
	H-**D** A Y M T M K I R N - OH	1242.5
	H-**βA**-Y M T M K I R N - OH	1127.4
	H-**Cha**- Y M T M K I R N - OH	1209.5
	H-A - **Pya** - M T M K I R N - OH	1113.4

H-A Y - **Met(O)** - T M K I R N -OH		1141.4
H-A Y - **Nle** - T M K I R N - OH		1109.4
H-A Y - **Nva** - T M K I R N - OH		1095.3
H-A Y M T - **Nle** - K I R N -OH		1109.4
H-A Y M T - **Nva** - K I R N -OH		1095.3
H-A Y M T M - **Orn** - I R N - OH		1113.4
H-A Y M T M - **Dab** - I R N - OH		1099.4
H-A Y M T M K - **Cha** - R N - OH		1167.5
H-A Y M T M K I **K** N - OH		1099.4
H-A Y M T M K I R N - **NH_2**		1126.4
H-A Y M T M K I R **n** - OH		1127.4
H- **a y m t m k i r n** - OH		1127.4
- **C** - A Y **L** T L K **I** R N - **C** -	Cyclic	1294.6
H-A Y M T M K I R N - OH	original peptide	1127.4
acet A Y M T M K I R N - OH		1169.4
H- **a** - Y M T M K I R N - OH		1127.4
H-A - **y** - M T M K I R N - OH		1127.4
H-A Y - **m** - T M K I R N - OH		1127.4
H-A Y M T - **m** - K I R N - OH		1127.4
H-A Y M T **I** K I R N - OH		1109.4
H-A Y M T M K - **M(ox)** - R N - OH		1161.4
H-A Y M T M K **M** R N - OH		1145.4
H-A Y M T M K - **i** - R N - OH		1127.4

	H-A Y M T M K - I - R E - OH	1142.4
dimer	$H_2NCH(CH_2CO\text{-}AYMTMKIRN\text{-}OH)_2$	2368.3

A= L-alanine, Y= L-tyrosine, M=L-methionine, T=L-threonine, K=L-lysine, I=L-isoleucine, R=L-arginine, N=L-asparagine, m=D-methionine, a=D-alanine, y=D-tyrosine, t=D-threonine, k=D-lysine, i=D-isoleucine, r=D-arginine, n=D-asparagine, MEA = methoxyethylamin (peptoid), 1BUA = 1-butylamin (peptoid), βA = beta-alanin, Cha = cyclohexylalanin, Pya = pyridylalanin, Met(O)= methionin-S-oxid, Nle = norleucin, Nva = norvalin, Orn = ornitin, Dab = 2,4-diaminobutyric acid.

E = L- D = L-

The dimere peptide was synthetized according to the article: Ligand Presenting Assembly. A Method for C- and N- terminal antigen presentation. A, Holm, R. M. Jørgensen, S. Østergaard, and M. Theisen. J. Peptide Res. (2000) **56**, 105-113.

The LPA technique makes it possible to couple the free α-amino groups at the amino terminal part of two IT9302 peptides together, while the two fully side chain protected peptide chains with a dicarboxylic acid are still attached to a synthetic resin.

Solubility test:

Portions of around 1mg af the peptides were weighed and dissolved in 1mg / ml PBS pH 7.4 buffer saline and were kept at -80 °C over night. Thereafter a sample of 100 µl was taken out and analyzes for its content of Alanine (or an other amino-acid), in order to determine the solubility of the peptides. After the first trial, the concentration tests showed that several of the synthetic peptides were difficult to dissolve, so we decided to add 10µl DMSO (Dimethylsulfoxid, Merck 1.02931) to each peptide for dissolving the aggregates, before adding PBS pH 7.4. Then the concentration test was made again by Professor Arne Holm.

IRAP production ng/ml	Test 1	Test 2	Test 3
Non stimulated cells	17.2	14.0	13.1
Cells stimulated with 100 ng / ml rIL-10	24.2	20.5	17.5
Analogues with ekvimolar concentration to rIL-10 ,were added 100 ng / ml			
H-**MEA** - Y M T M K I R N - OH	18.6	24.8 *	13.8
H-**E** - **MEA** - Y M T M K I R N -OH	20.1	18.1	14.9
H-**1BUA** - Y M T M KI R N - OH	22.1 *	23.2 *	17.7
H-**E** - **1BUA** - Y M T M K I R N - OH	21.8 *	22.5 *	19.4 ***
H-**E** A Y M T M K I R N - OH	18.1	21.9 *	19.7 *
H-**D** A Y M T M K I R N - OH	20.7	18.7	17.0
H-**βA**-Y M T M K I R N - OH	18.6	20.4 *	
H-**Cha**- Y M T M K I R N - OH	20.7	21.5 *	
H-A - **Pya** - M T M K I R N - OH	18.5	23.0 *	18.7 *
H-A Y - **Met(O)** - T M K I R N -OH	18.2	19.7	
H-A Y - **Nle** - T M K I R N - OH	20.0	21.8 *	13.9
H-A Y - **Nva** - T M K I R N - OH	21.5 *	21.9 *	
H-A Y M T - **Nle** - K I R N -OH	18.3	21.1 *	15.3
H-A Y M T - **Nva** - K I R N -OH	20.0	22.1 *	30.7 ***
H-A Y M T M - **Orn** - I R N - OH	22.5 *	21.3 *	14.7
H-A Y M T M - **Dab** - I R N - OH	24.2 *	21.1 *	
H-A Y M T M K - **Cha** - R N - OH	22.2	14.4	15.5

H-A Y M T M K I **K** N - OH	20.4	13.3	
H-A Y M T M K I R N - **NH_2**	27.9 ***	13.6	
H-A Y M T M K I R **n** - OH		14.2	
H- **a y m t m k i r n** - OH		13.6	
- **C** - A Y L T **L** K **I** R N - **C**		14.0	
H-A Y M T M K I R N - OH **(IT9302)**	23.7 *		18.1
acet A Y M T M K I R N - OH		14.7	
H- **a** - Y M T M K I R N - OH	19.5	13.7	
H-A - **y** - M T M K I R N - OH	19.0	14.1	
H-A Y - **m** - T M K I R N - OH	22.3	15.7	13.4
H-A Y M T - **m** - K I R N - OH	20.0	21.4 *	
H-A Y M T **I** K I R N - OH	20.9	14.7	
H-A Y M T M K - **M(ox)** - R N - OH	21.7	13.5	10.4
H-A Y M T M K **M** R N - OH		15.3	
H-A Y M T M K - **i** - R N - OH	20.6	15.6	
H-A Y M T M K - **I** - R **E** - OH	22.5 *	15.2	
H_2 NCH (CH_2CO-AYMTMKIRN-OH)$_2$			

Comments: Test 1. was carried out without addition of 10 µl DMSO. To avoid individual variation for each test, blod from the same donor person was used, one buffy coat of citrate blood. (*) marked values were the highest in the group, compared with rIL-10.

Extra measurements

IRAP production ng/ml		Test 4
Non stimulated cells		15.8 ± 0.9
Cells stimulated with	1ng/ml rIL-10	22.3 ± 0.6
	10 ng/ml	44.0 ± 0.3
	100 ng/ml	25.7 ± 0.4

Analogues with ekvimolar concentration to rIL-10

H-**MEA** - Y M T M K I R N - OH	1 ng/ ml	12.4 ± 1.4
	10 ng/ml	20.2 ± 0.7
	100 ng/ml	21.2 ± 1.1
H-**1BUA** - Y M T M K I R N - OH	1 ng / ml	16.0 ± 0.5
	10 ng / ml	19.5 ± 0.3
	100 ng/ml	19.6 ± 0.9
H-**E-1BUA**-YMTMKIRN - OH	1 ng/ml	14.9 ± 0.1
	10 ng/ml	24.0 ± 0.4 *
	100 ng/ml	19.5 ± 1.2
H-**E** A Y M T M K I R N - OH	1 ng/ml	16.8 ± 0.6
	10 ng/ml	22.0 ± 0.3 *
	100 ng/ml	20.9 ± 0.6
H-A - **Pya** - M T M K I R N - OH	1 ng/ml	14.9 ± 0.3
	10 ng/ml	20.6 ± 0.1
	100 ng/ml	21.9 ± 1.4

H-A Y M T - **Nva** - K I R N - OH	1 ng /ml	18.3 ± 0.9
	10 ng/ml	20.1 ± 0.4
	100 ng/ml	19.8 ± 1.2
H-A Y M T - **m** - K I R N	1 ng/ml	
	10 ng/ml	20.9 ± 0.6
	100 ng/ml	22.7 ± 0.4 *
$H_2NCH(CH_2CO\text{-}AYMTMKIRN\text{-}OH)_2$		
	1 ng/ml	14.9 ± 1.3
	10 ng/ml	20.3 ± 0.7
	100 ng/ml	21.2 ± 0.5 *
H-A Y M T K I R N -OH **(IT9302)**	1 ng/ml	23.6 ± 0.5
	10 ng/ml	28.6 ± 1.1 *
	100 ng/ml	28.6 ± 1.0 *

Comments: Test 4 was carried out with addition of 10 µl DMSO, but without making the concentration test.

Conclusion.

Based on the three first test with IRAP induction, we proposed 6 analogues which were minimum as potent as the original peptide. These were:

H - A Y M T M K I R N - OH original peptide

$- x_1 x_2 x_3$ T x_4 K x_5 R x_6 -

H-1Bua-Y M T M K I R N-OH

H-E-1Bua-Y M T M K I R N -OH

H-E-A Y M T M K I R N - OH

H-A -Pya- M T M K I R N -OH

H-A Y M T -Nva- K I R N -OH

H-A Y M T -m- K I R N -OH

Professor A. Holm made the following conclusion:

Met i position x_1 can be substitited with norvalin Nva as an unnatural aminoacid. Also Tyr (Y) in position x_2 shows the same possibility. This substitutions may bring stability against protease activity. At thc N-terminal part there are special possibilities for substitution. Ala (A) can be exchanged with 1Bua which is N-butyl-glycin or with glutamic acid-1Bua (E-1Bua). This substitution may bring stability against peptidase activities. The modification with 1Bua also propose that analogues which are more lipofile may be prefered. A lipofile analogue may stay for a longer time at the application site and thereby prolonge the activation time.The question about the C-terminal stabilization is not yet solved.

At the end a IT9302 dimer was also synthetized for the aim of stabilization. The dimere peptide shows the same minmum level of activity as the 6 choice of analogues.

The IL-10 and the IL-10 Receptor binding sites.

The crystal structure of human IL-10 and its soluble receptor IL-10Rα showed a IL-10 dimer binding two soluble receptors A. Zdanov et al (1996) Protein Science 5: 1955-1962.

Later on a second IL-10 receptor was discovered IL-10Rβ which was an essential subunit of the IL-10 receptor S.D. Spencer et al (1998) J.Exp. Med. vol.187, No.4 571-578.

Mapping the IL-10 /IL-10 receptor sites showed that the COOH terminal part is binding to the IL-10 Rα subunit.U. Reineke et al (1998) Protein Science, 7: 951-960.

A human IL-10 monomer was designed and this showed in contrast to the wilde type of IL-10 1:1 complexes with the soluble IL-10R (Rα). The binding of the IL-10 monomer to IL-10 Rα was sufficient for recruiting the signal transduction receptor chain (IL-10Rβ) into the signal complex and eliciting IL-10 cclular responses.K. Josephson et al. (2000) Vol. 275, No. 18, 13552-13557.